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The microspore development and haploid embryogenesis of anther culture with five nitrogen doses to the donor tobacco plants[1]

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ABSTRACT : By growing *N. tabacum* plants (Wisconsin 38) with different levels of nitrogen in basic Hoagland water solution, it was found that the amount of nitrogen supply can strongly influence the physiological status of the donor plants and thus the embryogenic ability of cultured anthers. Highest embryogenic ability was obtained when donor plants were grown in culture solution containing 15 mM nitrogen. Lower nitrogen concentration reduced the rate of embryo formation, whereas anthers produced from culture solution without nitrogen supply showed the poorest embryogenesis. Histological examination indicated that anthers derived from low nitrogen solution contained more highly-vacuolate and abortive microspores. The duration neeeded for the formation of microspores with starch granules and densely stained cytoplasm was less in optimal than in low nitrogen concentration. A strong and positive correlation was found between the number of starch-filled microspores and the number of embryos produced in culture. It is postulated that the starch-filled microspores might be able to provided nutrients to the developing embryos.

The culture of isolated plant organs, tissues, cells and protoplasts has attracted members of various fields in biology. Tissue culture technique is convenient not only for studying problems in physiology and biochemistry [21], but also for revealing the problems in cytogenetics and embryology [13,17]. In recent years, *in vitro* culture methods have found practical applications in genetic and breeding works. One of the most interesting approaches for breeders is anther culture for producing haploid plants.

The way in which donor plants are grown can strongly influence the haploid embryogenesis. Considerable evidences have supported the view that physiological status of the donor plants and environmental conditions can influence the production of haploid plants from anther culture [2,3,4,14,19]. Nitrogen supply is generally known of paramount importance in maintaining plants in a vigorous and healthy condition. It is reasonable to assume that it may also influence the haploid embryogenesis. My previous paper [20] reported that tobacco anthers obtained from donor plants grown in culture solution

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without nitrogen supply showed poor development of tapetum layer and the cytoplasm of microspores was lightly stained. I have proposed that fully-developed tapetum layer and microspores with densely stained cytoplasm are important for haploid embryogenesis in tobacco anther culture [20]. The objective of this study is to determine the relationship between nitrogen supply to the donor plants and embryogenetic characteristics of the excised anthers. The histological basis of haploid embryogenesis influenced by nutrient deficiencies has also been studied.

Materials and methods

Plants of *N. tabacum* L. cv. Wisconsin 38 grown in modified Hoagland culture solution [5] with various nitrogen levels [20] were used in this study. Anthers at mitosis microspore developmental stage were taken from the donor plants and were cultured on Murashige and Skoog (1962) medium as previously described [20].

Five anthers were sampled randomly from each of the five treatments (0, 1.5, 4.5, 15.0 & 45.0 mM nitrogen [20]) after 4, 7, 10, 14, 17, 21 and 24 days in culture and were fixed in FAA for at least 24 hours for histological study. After fixation, anthers were dehydrated in TBA series and embedded in paraplast. Sections were cut at 10 μm thickness on a rotary microtome with a steel knife, stained in Heidenhain's iron hematoxylin series and examined under a light microscope.

Results

A. Anther culture

The results in Table 1 showed that there was no significant difference in percentage of embryogenic anthers among 1.5, 4.5, 15.0 and 45.0 mM nitrogen treatments. However, 0 mM nitrogen treatment showed poor anthers embryogenesis as compared with the other four treatments. Either an excess or a deficient nitrogen supply in culture solution decreased the number of plants developed from cultured anthers. Culture solution having low nitrogen level lowered the embryogenic ability. Anthers grown in culture solution containing no nitrogen had the poorest embryogenesis.

Table 1. Anther culture of *N. tabacum* with five nitrogen concentrations applied to donor plants

Nitrogen in culture solution —mM—	Number of anther cultured	Embryogenic anthers			Total plants produced	No. of plants per	
		No.	%	0.95 CI		embryogenic anther	culture anther
0.0	39	15	38.5	24—57	289	19.5±6.6	8.9±2.4
1.5	181	134	74.0	66—81	2,732	20.4±2.6	15.1±2.0
4.5	177	102	57.6	50—66	2,434	23.9±2.6	13.8±2.4
15.0	181	119	66.1	58—72	4,277	35.9±3.2	23.8±2.9
45.0	177	104	58.8	51—67	3,564	34.3±3.2	20.1±27

B. Histological examination

a. Normal embryogenesis in cultured anthers

The normal embryogenesis of cultured anthers from donor plants grown in 15 mM nitrogen culture solution was shown in Fig. 1. This figure showed that microspores

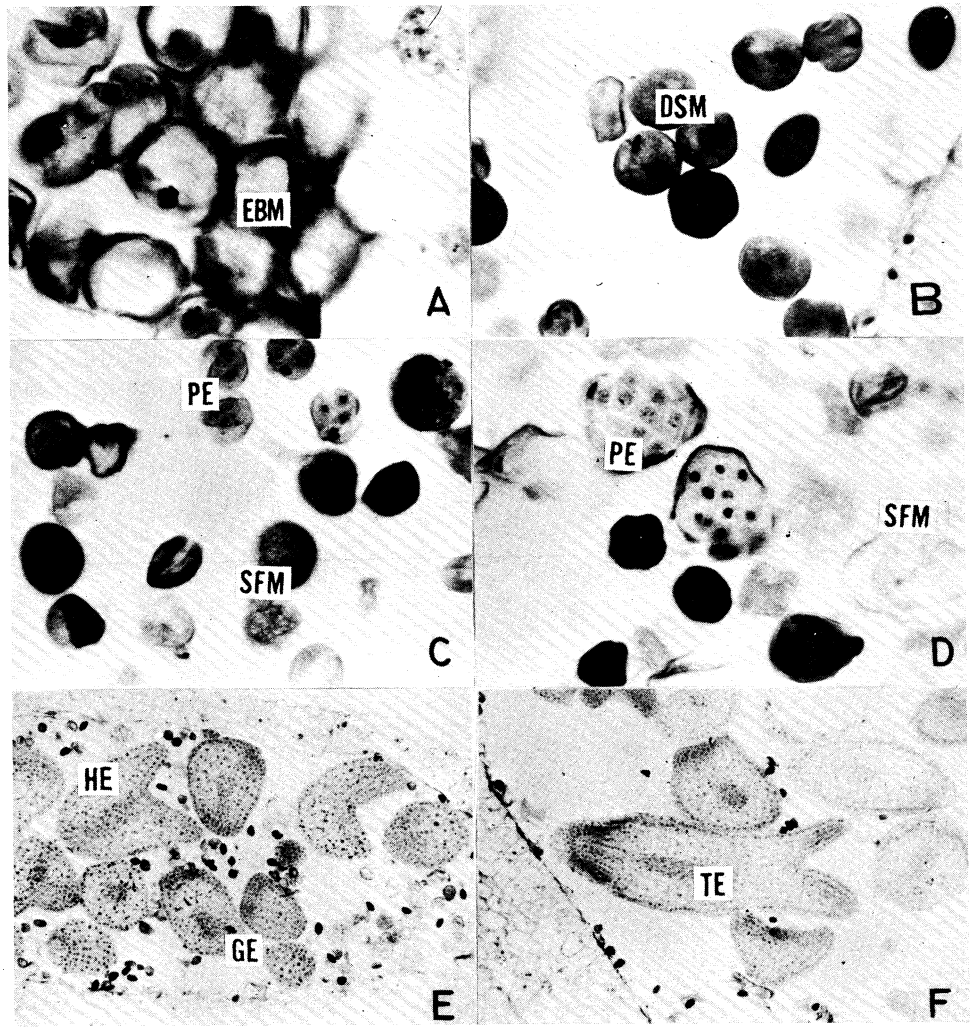

Fig. 1. Stages of embryogenesis in anther culture of *N. tabacum*. A. 4 days in culture, equal-sized binuclear microspores (EBM) were present. B. 7 days in culture, densely stained cytoplasm (DSM) in some microspores. C. 14 days in culture, starch grains in some microspores (SFM) and small pro-embryos (PE)observed. D. 17 days in culture, the starch-filled microspores (SFM) ruptured and embryos were growing. E. 21 days in culture, globular (GE) and heart (HE) shaped embryos observed. F. 24 days in culture, torpedo (TE) shaped embryos present. Magnification : A-D 55OX, E-F 225X.

with densely stained cytoplasm after 7-10 days in cultured. The starch-filled microspores and proembryos were formed 14 days later. Histological observation of anther embryo-

genesis revealed that both proembryos and starch-filled microspores might have derived from those microspores with densely stained cytoplasm. The starch-filled microspores did not generate embryo and degenerated later. It is then hypothesized that the starch-filled microspores might provide nutrients to the developing embryos. Table 2 indicated that

Table 2. The correlation between the number of starch-filled microspores and the number of embryos formed in anther culture of *N. tabacum* cv. Wisconsin 38

Days in culture	Nitrogen in culture solution —mM—	Starch-filled microspores —%—	Embryos —%—	Number of microspores studied
14	0.0	4.18	0.30	3,005
	1.5	8.88	2.41	2,860
	4.5	13.41	0.11	1,850
	15.0	0.47	0.04	2,743
	45.0	4.29	0.48	2,310
17	0.0	5.37	0.63	1,733
	1.5	11.63	3.84	2,890
	4.5	38.52	6.00	2,100
	15.0	22.31	4.66	2,537
	45.0	35.01	4.80	2,145
21	0.0	1.46	0.16	2,468
	1.5	22.73	3.57	2,275
	4.5	13.20	3.48	2,315
	15.0	28.23	4.65	3,465
	45.0	27.64	7.88	1,650

the more starch-filled microspores were present, the more embryos formed. A linear correlation ($r=0.86^*$) was found between the percentages of starch-filled microspores and embryos present in tobacco anthers (Fig. 2).

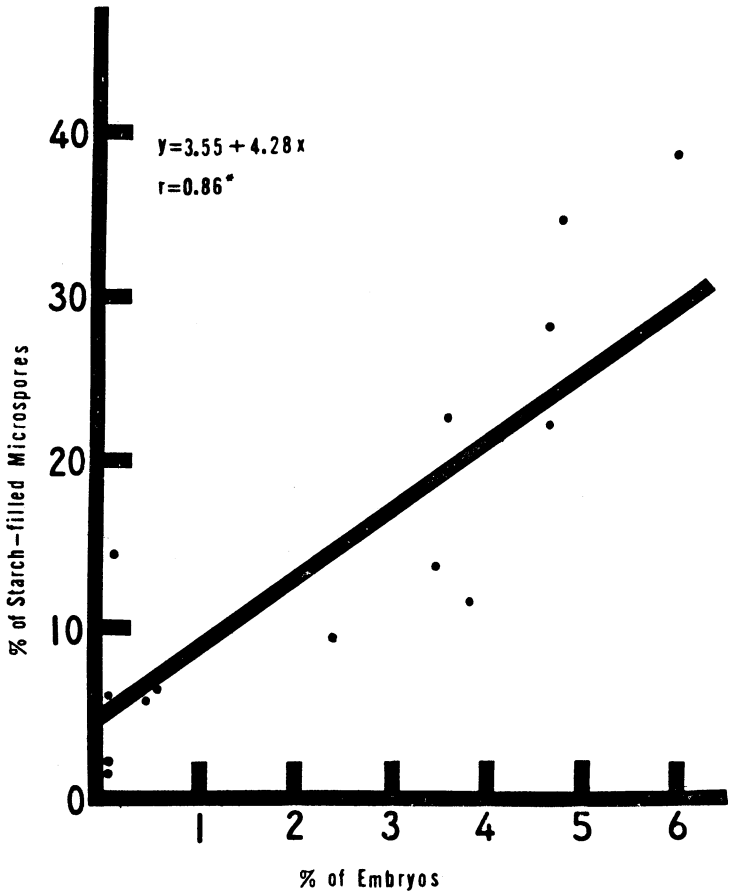

Fig. 2. The regression line between numbers of starch-filled microspores and embryos. Data obtained from tobacco anthers cultured for 14. 17 and 21 days.

In general, the starch-filled microspores were much bigger than the ordinary ones. They often bursted out of the exine but not the intine. They usually have vacuole and 2-3 nuclei. Figure 3A showed the formation of starch-filled microspores 14 days after anther culturing. The microspores ruptured after 17 days (Fig. 3A)

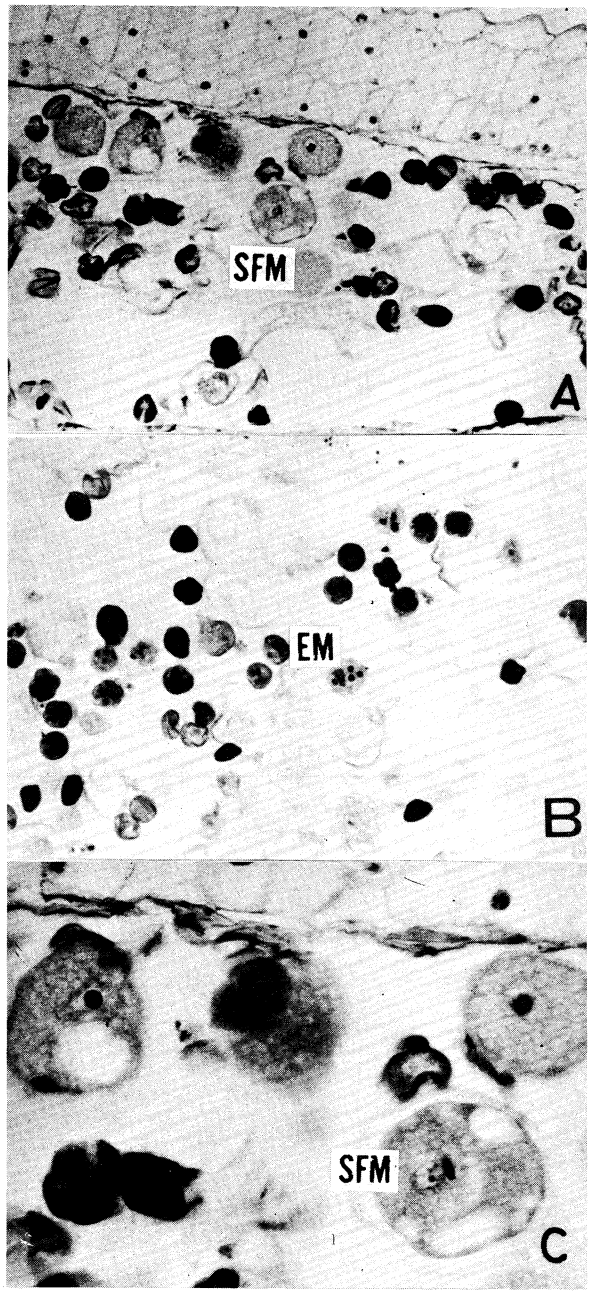

Fig. 3. The formation of starch-filled microspores. A. Starch-filled microspores (SFM) formed after anthers were cultured for 14 days. B. After 17 days in culture showing the ruptured starch-filled microspores and the embryos (EM). C. Starch-filled microspores in high magnification. Magnification : A-B 550X, C 2500X.

b. Normal embryogenesis in high nitrogen treatments and deviations caused by low nitrogen supply.

Table 3 showed that for 0 mM nitrogen treatment, anthers produced many abortive

Table 3. The effect of nitrogen supply to the donor plant (*N. tabacum* cv. Wisconsin 38) and the formation of dead microspores in anther culture

Days in culture	Nitrogen in culture solution —mM—	Dead microspores —%—	Number of microspores studied
10	0.0	61.69	2,480
	1.5	39.85	3,360
	4.5	50.43	3,020
	15.0	36.07	5,367
	45.0	37.72	2,731
14	0.0	64.23	3,005
	1.5	39.76	2,860
	4.5	30.57	1,850
	15.0	46.66	2,743
	45.0	33.81	2,310
17	0.0	67.05	1,733
	1.5	57.06	2,890
	4.5	34.81	2,100
	15.0	54.43	2,537
	45.0	40.05	2,145
21	0.0	60.49	2,468
	1.5	59.11	2,275
	4.5	58.09	2,315
	15.0	47.50	3,465
	45.0	49.39	1,650
24	0.0	83.69	1,846
	1.5	78.40	2,060
	4.5	46.84	1,665
	15.0	37.42	1,510
	45.0	57.20	1,849

microspores with clear cytoplasm after 10 days in culture (Fig. 4). For 1.5 and 4.5 mM nitrogen treatments, many highly-vacuolated microspores were found in anthers of 4-days-culturing (Fig. 4B-C). After anthers were cultured for 21 days, all nitrogen deficient treatment (0, 1.5 and 4.5 mM nitrogen) produced more abortive microspores and smaller embryos as compared to 15 and 45 mM nitrogen treatments, as shown in Fig. 4D-F and Table 3.

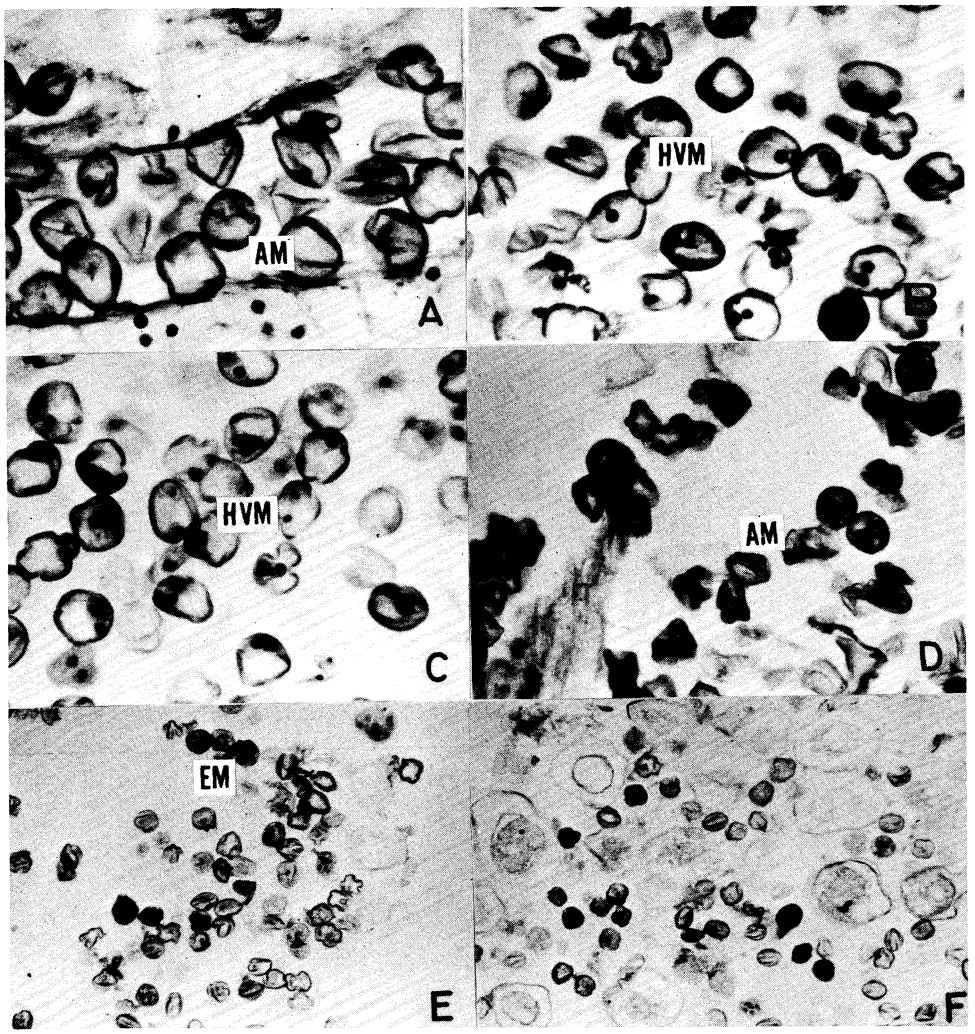

Fig. 4. The deviation from normal embryogenesis in low nitrogen treatments. A. The abortive microspores of O mM nitrogen treatment after 14 days in culture. B-C. The highly vacuolated microspores (HVM) in anthers of 1.5 (B) and 4.5 (C) mM nitrogen treatments, after 4 days in culture. D-F. High frequencies of abortive microspores (AM) and small embryos (EM) with 0 (D), 1.5 (E) and 4.5 (F) mM nitrogen treatments after 21 days in culture. Magnification : A-D 1400X, E-F 550X.

There were also differences in formation of microspores with densely stained cytoplasm and starch-filled microspores among five treatments. The time needed for the formation of microspores with starch granules and stained cytoplasm in cultured anthers differed among nitrogen treatments. The lower the nitrogen was supplied ; the longer the time needed (Table 4). In high-nitrogen treatment, both the densely stained cytoplasm and starch-filled microspores were found in culture of only 4 days.

Table 4. The effect of nitrogen supply to the donor plants on the formation of microspores with densely stained cytoplasm, microspores with two identical nuclei and starch-filled microspores in anther culture of *N. tabacum* cv. Wisconsin 38

Days in culture	Nitrogen in culture solution —mM—	Microspores		Starch-filled microspores —%—	No. of microspores studied
		with densely stained cytoplasm —%—	with two identical nuclei —%—		
4	0.0	—	—	—	—
	1.5	0	0.84	0	3,710
	4.5	0	0.82	0	4,380
	15.0	0	3.38	0	6,900
	45.0	9.77	1.06	1.15	5,116
7	0.0	0	0.92	0	4,651
	1.5	0	1.22	0	4,346
	4.5	6.14	2.07	0	3,094
	15.0	13.48	2.06	0	6,513
	45.0	6.03	1.04	2.74	5,470
10	0.0	0.28	0.32	2.06	2,480
	1.5	1.99	0.15	7.74	3,360
	4.5	13.18	0.20	5.30	3,032
	15.0	5.20	1.57	0.22	5,367
	45.0	14.71	0.26	1.10	2,731
14	0.0	7.69	0	4.13	3,005
	1.5	8.22	0	8.88	2,860
	4.5	11.19	0.32	13.41	1,850
	15.0	8.60	0.73	0.47	2,743
	45.0	4.42	0.13	4.29	2,310

Discussion

Histological study revealed that anthers from plants grown in low nitrogen culture solution contained more highly-vacuolated and abortive microspores. The duration needed for the formation of microspores with densely stained cytoplasm and starch-filled microspores was shortened when the donor plants were properly supplied with nitrogen. The most dramatic difference between the non-embryogenic and embryogenic anthers was the formation of starch-filled microspore which occurred as early as 4-10 days in culture in the embryogenic anthers. In contrast, there was hardly any starch-filled microspore in the non-embryogenic anthers.

The question posed by the foregoing is whether the formation of starch-filled microspore plays a causative role in embryo formation, or whether it is just an end product of cellular metabolism, unrelated directly to the embryo initiation process. Because of the following reasons, it appears reasonable to infer that starch-filled microspores play a definite role in the embryo initiation process in tobacco anther culture. First of all, the formation of starch-filled microspores proceded any observable embryos. The formation started in 4 to 10-day-old cultured anthers, and this was before the formation of embryos which were not observed until after 14 days. Secondly, no embryos were formed in cultured anthers lacking starch-filled microspores. In addition, the starch-filled microspores of embryogenic anthers ruptured and released their content between 17 and 21 days of culture. These observations suggest that starch is used both during embryo initiation and the subsequent developmental stages. Studying cell development during cotton embryogenesis, Jensen (1963) observed the presence of a large number of starch-containing plastids in the synergids and polar nuclei of mature gametophyte. During the early stages of embryo development, there was abundant starch in the zygote, but as it divided, the amount of starch per cell decreased [6]. Thorpe and Murashige (1970), in studying organ formation of tobacco stem pith callus, found that the difference between the non-organ-forming and organ-forming tissues was the accumulation of starch in the latter. They reported that GA_3-treated tissues showed the absence of organ formation and a marked reduction in accumulated starch and interpreted that GA_3 caused the *de novo* synthesis of α-amylase which in 4 to 5 days reached a sufficently high concentration to hydrolyze the starch formed. Thus, if a certain concentration of starch is required to support the organ initiation activities, GA_3 prevented this concentration from being reached, and thereby inhibited shoot formation [18].

Thirdly, the conclusion that starch-filled microspore was important to embryo formation and embryo development was supported by the data obtained from anther cultured for 14, 17 and 21 days. There was a positive correlation between the number of starch-filled microspores and the number of embryos. Finally, in other organogenetic processes starch accumulation has been a conspicuous feature. Marinos (1967) studied the growth of potato buds following the breakage of dormancy. He found that the starch concentration was low in cells of the shoot apex and young leaves, but was very great in the underlying parenchyma cells [7]. Sadik and Ozbun (1967) observed a direct relationship between the presence of starch and floral induction in cauliflower. They found that shoot apices of vegetative plants contained little or no starch. However, the shoot apices of plants induced to flower accumulated large amounts of starch. They also found that floral primordia which developed into functional flowers had very heavy deposits of starch, while floral primordia which aborted were devoid of starch [12].

The question arises, therefore, about the physiological significance of starch-filled microspore in the embryogenic process. Two functions of starch-filled microspore appear possible. Firstly, the formation of starch-filled microspore and its subsequent use during embryo development may reflect the high energy requirement of the embryogenic process. Jensen (1963) found a continued increase in oxygen uptake with the development of the

cotton embryo, while there was a decrease in the amount of starch present[6]. As a source of energy, starch possesses a distinct advantage. Its degradation by phosphorylase, now considered the most probable mechanism, would result in high yields of glucose-1-phosphate, the subsequent metabolism of which through glycolysis would produce substrate-level adenosine triphosphate (ATP), without the expenditure of high energy phosphates at that time. The degradation of pyruvate through the TCA cycle and concomitant oxidative phosphorylation would lead to additional ATP production. The high-energy phosphates produced would then be available for cellular metabolism[18]. The second possible role of starch in the organogenetic process is osmotic. The degradation of starch to oligo-saccharides could effectively and systematically increase the osmotic potential of the cells and thus lead to increased water uptake. This increases in water uptake would keep proteins fully hydrated and allow maximum enzyme activity[18].

The formation of starch-filled microspores in anther culture has been reported by many authors. Sunderland[16], Sunderland and Wicks[17], and Bernard[1] observed that in anther culture of *N. tabacum*, certain pollen grains enlarged as a result of starch deposition and they often bursted out of the exine, thus giving rise to giant bodies enclosed by the intine. These giant bodies had 2 or 3 nuclei and they did not undergo embryogenesis[1,14,15,16,17]. Rashid and Street[11] reported that in anther cultures of *Atropa belladonna*, anthers which failed to produce embryos always contained abnormally enlarged pollen packed with starch grains. However, these abnormal pollen grains were also observed in anthers producing embryo. From the observation of the histological study, it was found that although the starch-filled microspores did not actually undergo embryo formation, they might serve as a readily availably source of energy for the initiation of embryos and the subsequent development of these embryos, both activities presumably have very high-energy requirements.

It is obvious that the role of nitrogen supply is critical in haploid embryogenesis in tobacco. The influence of P, K, and other elements should also be examined by using the same methods. These studies may also be expanded to explore how pathogens, e. g., virus, would affect plants and consequently the success of anther culture.

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氮素用量對於草培養花葯小胞子的發育與單倍體之形成[1]

蔡 新 聲[2]

摘 要

於草以改良 Hoagland 基本鹽類的水耕液培養，發現氮素不同量的供給能左右母體植物生長的好壞，進而影響到由此植物所取花葯培養單倍體的形成。花葯培養的結果顯示低濃度氮素量生長下的母體植物（尤其是不含氮素量處理區），其單倍體之形成較差。花葯培養後不同時期的組織切片顯示，適當氮素濃度生長下之母體植物，其花葯培養後形成「細胞質染色力較强之小胞子」及「澱粉粒小胞子」所需的時間均較低氮素量生長下母體植物之花葯爲早；由於「具有澱粉粒小胞子」的數目與胚狀體形成的數目有正相關，所以母體植物生長於不同氮素量下，可間接左右花葯培養後「具有澱粉粒小胞子」及「細胞質染色力較强小胞子」形成的早晚，進而影響到單倍體形成的能力。切片的觀察顯示「具有澱粉粒小胞子」的功能爲瓦解後供給發育中胚的養分。又低氮素量生長下所得之花葯，經培養後形成之高度空泡化小胞子及退化小胞子的數目亦均比母體植物生長於適當氮素量下所得之花葯培養爲多。

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